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A process for the extraction of poly-3-hydroxy-butyric acid from microbial cells.

Abstract:

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54 **A process for the extraction of poly-3-hydroxy-butyrac acid from microbial cells.**

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## TITLE MODIFIED

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Extraction process

This invention relates to an extraction process and in particular to a process of extracting poly-3 -hydroxybutyric acid, hereinafter referred to as PHB from microbial cells.

5 It has been known since the 1920's that many micro-organisms are capable of accumulating granules of PHB within their cells as an energy reserve material. It has been proposed in USP 3107172 to dry such PHB containing bacterial cells, for example by spray drying, and to use the resultant dried cells as moulding compos-  
10 itions. Suggestions have also been made to extract PHB from bacterial cells and to use it as a plastics material, but methods so far disclosed have not been economically acceptable.

In order to extract the PHB, it is generally necessary to contact the bacterial cells with a solvent in which PHB is  
15 soluble to leach out the PHB from the remainder of the bacterial cell material. Some bacteria, for example members of the genus Azotobacter readily yield up their PHB to the extraction solvent, whereas other bacteria, eg Pseudomonadaceae have more robust cells and require a cell disruption step prior to contact with the ex-  
20 traction solvent.

Methods of extraction previously proposed have included the steps of harvesting the bacterial cells from the aqueous fermentation medium, eg by centrifugation, to give a mass of wet cells which are then contacted with acetone to effect drying and cell  
25 breakage. After removal of the acetone, the PHB is extracted with a suitable solvent, eg pyridine (USP 3036959) or a dichloromethane/

ethanol mixture (USP 3044942). Such methods have the advantage that in addition to effecting drying and cell breakage, acetone also extracts lipids and pigments (if any) which would otherwise contaminate the product. However treatment of a mass of wet cells with acetone to effect drying and cell breakage is not economic on a large scale.

Another method is described in USP 3275610 wherein a dispersion of bacterial cells in water is subjected to ultrasonic vibration to rupture the cells followed by centrifugation and drying before extraction with a solvent such as chloroform. After separation of the PHB from the chloroform solution, the PHB is washed to extract lipids therefrom.

It has also been proposed in USP 4101533 to extract PHB from dried cells, or directly from a wet mass of cells harvested from the fermentation medium by centrifugation, by heating the cells with certain cyclic carbonate solvents.

It is also possible to extract PHB directly from the aqueous cell suspension produced by fermentation, preferably after some concentration, by contact with certain solvents such as chloroform, dichloromethane, or 1,2-dichloroethane with, where necessary, a cell disruption step, eg milling, prior to contact with the solvent. However the solvent, and extraction conditions, have to be selected with care to avoid undue uptake by the solvent of non-PHB material particularly lipids and pigment (if any) present in the bacterial cell. Not only does such non-PHB material contaminate the product and so present purification difficulties but also the co-extraction of lipids may tend to result in the formation of a relatively stable emulsion between the solvent and aqueous phases rendering separation thereof difficult. With such a direct extraction process, a separate lipid extraction step prior to contact with the PHB extraction solvent is generally not practical as the solvents that extract lipids would need to be removed, together with the lipids, prior to contact with the PHB extraction solvent and, because the more effective lipid solvents tend to be water miscible, such removal of the lipid solution presents practical

difficulties.

We have now found that PHB can be extracted from bacterial cells by a particularly simple process amenable to large scale operation.

5 According to the present invention we provide a process for the extraction of PHB from an aqueous suspension of PHB-containing bacterial cells comprising introducing said suspension in finely divided form into a current of gas heated to a temperature of at least 100°C to evaporate the water from said suspension, collecting  
10 the resultant dried bacterial cells, extracting the PHB therefrom by contact with an extraction solvent which is a liquid that is a solvent for the PHB in the bacterial cells, and separating the extraction solvent having the PHB dissolved therein from the bacterial cell residue.

15 We have found that such a drying process sufficiently weakens the bacterial cells to enable the PHB to be extracted without the necessity for any separate cell breakage step. While for particularly robust bacteria a separate cell breakage step, eg milling, prior to drying may be desirable to increase the yield of PHB  
20 extracted, we have found that generally such a separate cell breakage step prior to drying is not necessary and indeed is best avoided if possible as drying milled dispersions gives rise to difficulties such as build-up in the drier.

In the process of the invention it is preferred to subject  
25 the cells to a lipid extraction step prior to extraction with the PHB extraction solvent. Thus the dried cells may be extracted with a lipid/pigment solvent such as acetone, methanol, ethanol, butanol, hexane, or petroleum ether, followed by separation of the solvent containing dissolved lipids/pigment from the cells prior to contact-  
30 ing the cells with the PHB extraction solvent. The lipid/pigment extraction is preferably performed by refluxing the dried cells with the solvent. Acetone and methanol are the preferred lipid extraction solvents. The lipid/pigment extraction solvent may be used in admixture with another PHB non-solvent such as diethyl  
35 ether.



It will be appreciated that this lipid extraction may also give rise to some further weakening or breakage of the cells thus facilitating the subsequent extraction of the PHB.

In the process of the invention the cells are separated  
5 from the aqueous suspension by a drying process involving introducing the cell suspension in finely divided form, eg as a spray or fine stream, into a current of gas, eg air, heated to a temperature of at least 100°C. Preferably the suspension is introduced via a spray or atomising nozzle. Such drying processes are well  
10 known and include spray and flash drying.

The heated gas current evaporates off the water which is carried away by the gas stream leaving the dried cells which are collected for extraction with the PHB extraction solvent.

The gas inlet temperature may be in the range 100°C to  
15 500°C and is preferably in the range 120°C to 250°C.

Suitable PHB extraction solvents include pyridine, cyclic carbonates and, particularly, partially halogenated hydrocarbons such as chloroform, dichloromethane and 1,2-dichloroethane. 1,2-Dichloroethane is not normally considered to be a solvent for PHB  
20 because PHB, after separation from bacterial cells, does not readily, or completely, dissolve in 1,2-dichloroethane. Thus, whereas the PHB solution separated from the bacterial cell residue appears to be a single phase, a solution made by re-dissolving PHB in 1,2-dichloroethane after precipitation is, except when very dilute,  
25 pearly in appearance, and PHB that has been precipitated and dried does not readily re-dissolve in that solvent. It is therefore surprising that such a solvent should be effective for extracting PHB from cells.

Not only is 1,2-dichloroethane a suitable solvent for  
30 use when extracting PHB from the dried cells but, as is described hereinafter, it may be used to effect extraction of PHB directly from an aqueous cell suspension under certain conditions with little contamination of the extracted PHB with co-extracted materials. Therefore according to a further aspect of the invention  
35 we provide a process for the extraction of PHB from bacterial cells

containing PHB comprising contacting the bacterial cells with 1,2-dichloroethane and separating the PHB containing solvent phase from the bacterial cell residue.

In the direct extraction process in which an aqueous cell suspension is contacted with 1,2-dichloroethane as the extraction solvent, after milling or other cell disruption step if robust bacteria are employed, the temperature of the extraction solvent should be below 40°C to avoid undue uptake of non-PHB material by the solvent. In contrast thereto, where the cells are dried as described hereinbefore before contact with the solvent for efficient extraction, the extraction is preferably conducted at a temperature above 40°C. Thus temperatures up to, and including, the solvent boiling point may be used and superatmospheric pressures may be employed to enable temperatures in excess of the solvent boiling point at atmospheric pressure to be employed.

Where, the cells are dried prior to contact with the extraction step and, as is not preferred for reasons described hereinbefore, the cell suspension is subjected to a cell disruption step, eg milling, prior to drying, the extraction temperature should be below 40°C to avoid undue uptake of lipids. Thus if a milled dispersion is dried and extracted with a hot solvent, on precipitation of the PHB from the solvent, a gelatinous sticky mass tends to be formed. Where however a lipid extraction step is employed prior to contact with the PHB solvent, the extraction with the PHB solvent may be conducted at temperatures above 40°C.

The weight of PHB extraction solvent used is preferably 10 to 100 times the cell dry weight. The use of smaller amounts of solvent may reduce the extraction efficiency of crude PHB and may give solutions of excessive viscosity while the use of larger amounts is uneconomic. The amount of solvent is preferably such that the extracted solution contains 0.5 to 5%, particularly 1 to 2% PHB by weight.

The contacting time for extraction should be a compromise to give adequate extraction without being uneconomically lengthy.

Where the cells are dried prior to contact with the



extraction solvent, separation of the cell residue from the PHB-containing solution may be effected by a simple filtration or centrifugation step. If the cells are subjected to a lipid extraction prior to extraction of the PHB, the filtration of the PHB solution from the cell residue tends to be particularly facile and can be effected using relatively coarse filters. Where the PHB is extracted by contacting an aqueous suspension of the bacterial cells with 1,2-dichloroethane, the bacterial cell residue may be removed by separating the 1,2-dichloroethane phase (which contains the dissolved PHB) from the aqueous phase: the bacterial residue remains suspended in the aqueous phase. It is sometimes difficult to separate the 1,2-dichloroethane phase from the aqueous medium as a stable emulsion may be formed. While in some cases centrifugation will facilitate separation of such emulsions, centrifugation is not always successful and anyway is not attractive in large scale operation.

We have found that separation of the solvent and aqueous phases may be facilitated by adjustment of the pH of the aqueous phase, prior to contact with the 1,2-dichloroethane, to within 0.5 units of pH of the isoelectric point of the bacterial cells. As will be mentioned hereinafter, for some bacterial cells, it is desirable to disrupt the cells prior to contact of the aqueous cell suspension with the 1,2-dichloroethane. The isoelectric point of the disrupted cells may well differ from that of the unbroken cells. Where the cells are disrupted the pH should be adjusted to within 0.5 units of pH of the isoelectric point of the disrupted cells. While it is possible to add sufficient of a pH modifying material to the suspension of cells prior to cell disruption so that, on disruption the pH is within 0.5 units of the isoelectric point of the disrupted cells, it is preferred to adjust the pH after the step of cell disruption.

The isoelectric point of a bacterial cell is the pH value at which the cell possesses no set positive or negative charge, ie it is electrically neutral. The isoelectric point may be determined by \_\_\_\_\_



particle electrophoresis measurements at differing pH values and hence the pH at which the net charge is zero is determined.

Generally for PHB-accumulating bacteria the isoelectric point, both for whole cells and for disrupted cells, is within the pH range 4 to 5.5; however the pH of the aqueous medium during fermentation will generally be within the range 6 to 8, the optimum depending on the nature of the bacteria used and the other fermentation conditions. As indicated above the pH of the aqueous medium should be adjusted to within 0.5 units of pH of the isoelectric point. Preferably it is adjusted to within 0.25 pH units of the isoelectric point and, more particularly, to a pH within the range of the isoelectric point to 0.25 pH units above the isoelectric point. It will be appreciated that such pH adjustment is not necessary where the cells are dried prior to contact with the PHB extraction solvent.

After separation of the PHB-containing extraction solvent from the bacterial cell residue, the PHB solution may be further filtered, if desired, to remove any suspended bacterial fragments. Such filtration is preferably conducted using a filter, eg a glass fibre filter, having a pore size of less than 5  $\mu\text{m}$ , preferably less than 2  $\mu\text{m}$ .

The separated PHB-containing solution can be used directly, preferably after filtration, for making solvent cast articles such as coatings, films or fibres or the solution may be treated further to separate solid PHB, for example by evaporation of the solvent or by precipitation by addition of the PHB-containing solution to a liquid in which PHB is insoluble and with which the solvent is miscible. Examples of suitable liquids include petroleum ether and methanol/water mixtures. The PHB may be purified, if desired, by washing with methanol or acetone.

After extraction of the PHB, the bacterial cell residues may be further refined for other uses, eg as a feedstuff or fertilizer.

Any bacteria that are capable of accumulating PHB may be used to produce the PHB-containing bacterial cells. A paper by Senior et al in *Advances in Microbial Physiology* 1973 10 203 - 266

lists the bacteria published up to June 1972 and others are described in US Patent 3072538 (Rhizobium mutants) and UK Patent 1535632 (especially mutants of Alcaligenes eutrophus, Bacillus megaterium, Zoogloea ramigera, and Mycoplasma rubra). Among the preferred bacteria are Azotobacter, especially chromococcum, Alcaligenes, especially eutrophus, and the Pseudomonadaceae, especially Pseudomonas AM 1 and Methylobacterium organophilum.

Among such bacteria are those capable of metabolising one or more of a variety of substrates, for example carbohydrates, ethanol, methanol, polyhydric alcohols, carbon dioxide/hydrogen, and carboxylic acids, and, according to the substrate used, may grow aerobically or anaerobically. The invention is of particular utility in separating PHB from bacterial cells of the Pseudomonadaceae grown under aerobic fermentation conditions on an alcohol, particularly methanol, substrate. The invention is also of particular utility in separating PHB from Azotobacter grown on a water soluble carbohydrate such as sucrose or glucose.

The cell suspension produced by the fermentation process will typically contain 20 to 55 g l<sup>-1</sup> biomass solids. Where the suspension is extracted with 1,2-dichloroethane or a pigment/lipid extraction solvent without an intermediate drying step, for efficient extraction, the cell suspension preferably has a concentration of 5 to 15% by weight biomass solids. The cell suspension is preferably concentrated, eg by centrifugation, to within this range where this is necessary. (The cell suspension as produced in the fermentation process may already have a concentration within this range: however even in such cases some concentration may be desirable).

We have found that, when extracting the PHB directly from the aqueous cell suspension with 1,2-dichloroethane, the PHB can be extracted from some bacteria without the need for a separate cell disruption step. Thus members of the genera Azotobacter and Alcaligenes readily yield up their PHB to the 1,2-dichloroethane solvent such that the extraction step requires only agitation of the cell suspension with the solvent. The agitator preferably

includes adjacent surfaces in relative movement thus providing mild shearing. A Silverson (RTM) blender may be used to effect mixing. More robust bacteria, eg Pseudomonadaceae require a distinct step of cell disruption. This disruption can be carried  
5 out by shearing the aqueous cell suspension, for example by homogenisation, bead-milling, roller-milling, or French pressing. Other methods of cell disruption include osmotic shock, sonic or ultrasonic vibration, and enzyme cell-wall lysis. Treatment with hypochlorite to destroy the bacterial cell membrane chemically  
10 could be used but preferably is not because it usually causes severe degradation of the PHB. Surprisingly we have found that spray or flash drying the cell suspension also sufficiently weakens the cells to allow the PHB to be extracted without any other cell disruption step. Hence no separate cell disruption step is  
15 required where the aqueous cell suspension is spray or flash dried prior to contact with the extraction solvent.

The invention is illustrated by the following examples in which all percentages are by weight.

EXAMPLE 1 (Comparative)

20 This example demonstrates that simple air drying does not weaken the cells sufficiently to allow efficient PHB extraction.

1000 ml of an aqueous suspension of Methylobacterium organophilum (NCIB 11483 - which is further described in our UK Patent Application 79 06078) containing 60 g biomass solids of  
25 which 36% was PHB was centrifuged to give a pellet of wet bacterial cells.

(NCIB No. refers to the number of the culture deposited at the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland).

30 The pellet was then dried in a fluid bed drier at 40°C for 10 hours.

10 g of the resultant dried cells were suspended in 500 ml of 1,2-dichloroethane at room temperature for 15 minutes and then the solvent phase was removed by centrifugation and decanting. This  
35 solution was added to 3000 ml of a methanol/water mixture (4 volumes

of methanol to 1 volume of water) with vigorous stirring to precipitate crude PHB. The precipitate was collected on a filter and dried in vacuo at 50°C. The yield of crude PHB was less than 0.5%.

The above experiment was repeated but the dry cells were sheared with the 1,2-dichloroethane in a Silverson mixer at room temperature for 15 minutes. The yield of crude PHB was 1.4%.

The above experiment was repeated but the dry cells were refluxed with the 1,2-dichloroethane at 83°C for 15 minutes. The yield of crude PHB (purity 94.5%) was 29%.

(Yields are calculated herein as  

$$\frac{\text{weight of crude PHB recovered}}{\text{dry weight of cells used}} \times \frac{100}{\% \text{ PHB in cells}} \times 100$$
)

#### EXAMPLE 2

5000 ml of the aqueous suspension of bacterial cells used in Example 1 was spray dried at a suspension feed rate of 5000 ml hr<sup>-1</sup>, an air inlet temperature of 150°C; an air outlet temperature of 80°C and an air flow rate of 300 m<sup>3</sup> hr<sup>-1</sup>.

20 g of the spray dried cells were suspended in 1000 ml of 1,2-dichloroethane at room temperature for 15 minutes. The cell debris was removed by filtration through a Whatman 541 paper filter. The PHB was recovered from the solution by adding the latter to 5000 ml of a methanol/water mixture (4 vol. methanol:1 vol. water) with vigorous stirring. The precipitate was collected on a filter. The yield of crude PHB was 5.7%.

The above experiment was repeated except that the dried cells were sheared with the 1,2-dichloroethane in a Silverson mixer at room temperature for 15 minutes. The yield of crude PHB (purity 98.2%) was 12.2%.

The above experiment was repeated except that the dried cells were refluxed with the 1,2-dichloroethane at 83°C for 15 minutes. The yield of crude PHB (purity 93.6%) was 98.3%. The precipitate was washed 5 times with 500 ml aliquots of methanol and then dried at 106°C. The purity of the washed PHB was 98.7%.

#### EXAMPLE 3

20 g of the spray dried cells as used in Example 2 were

refluxed for 5 minutes with 600 ml of acetone at 56°C to extract lipids and pigment and then the acetone removed by filtration. The residual cells were then sheared for 15 minutes at room temperature with 1000 ml of 1,2-dichloroethane in a Silverson mixer. The resultant solution was filtered from the cell residue using a Whatman 541 paper filter and the PHB was then precipitated by adding the solution to 5000 ml of a methanol/water mixture (4 vol. methanol: 1 vol. water) with vigorous stirring. The precipitate was collected on a filter.

10           The yield of crude PHB (purity 96.7%) was 43.2%.

The above procedure was repeated save that instead of shearing the acetone extracted cells with 1,2-dichloroethane, the acetone extracted cells were refluxed for 15 minutes with 1,2-dichloroethane at 93°C.

15           The yield of crude PHB (purity 98.4%) was 95%.

It is thus seen that while the acetone extraction further weakened the spray dried cells to allow more PHB to be extracted by cold 1,2-dichloroethane, spray drying alone sufficiently weakened the cells to permit efficient extraction of the PHB by boiling 1,2-dichloroethane. The acetone extraction did, however, improve the purity of the extracted PHB.

#### EXAMPLE 4

An aqueous suspension of cells of Azotobacter chroococcum (NCIB 9125) containing 60 g l<sup>-1</sup> biomass solids of which 37.8% was PHB was spray dried, acetone extracted, extracted with 1,2-dichloroethane under reflux, and precipitated using the conditions described in Example 3 above.

The yield of crude PHB (purity 98%) was 89.4%.

#### EXAMPLE 5

30           Example 4 was repeated using methanol instead of acetone as the lipid extraction solvent and dichloromethane instead of 1,2-dichloroethane as the PHB extraction solvent. The yield of PHB (purity 98%) was in excess of 95%.

Similar results were obtained using chloroform as the PHB  
35 extraction solvent.

EXAMPLE 6

A culture of Azotobacter chroococcum (NCIB 9125) was produced by fermentation of glucose in an aqueous medium under carbon-excess, oxygen-limitation, and minimal salts concentration conditions. The cell suspension contained  $20 \text{ g l}^{-1}$  biomass solids, the PHB content of which was 40%. The suspension was then concentrated to a cell cream containing  $80 \text{ g l}^{-1}$  biomass solids by centrifugation.

500 ml of the cell cream was mixed with 1000 ml of 1,2-dichloroethane at  $20^{\circ}\text{C}$  and blended for 10 minutes by means of a Silverson (RTM) blender, model L2R. Owing to energy dissipation the temperature rose to  $40^{\circ}\text{C}$ . The resulting emulsion was cooled and then centrifuged at  $13000 \text{ g}$  for 15 minutes at  $15^{\circ}\text{C}$ . The upper layer of aqueous solution and cell debris was decanted off. The lower layer, PHB-dissolved in 1,2-dichloroethane, was poured slowly with vigorous stirring into 5000 ml of a methanol/water mixture (4 volumes methanol:1 volume water).

The precipitate (purity 97.5%) was collected on a filter, washed five times with 1000 ml of methanol and dried in vacuo at  $50^{\circ}\text{C}$ . It was analysed chemically and found to have the following composition: C 55.8, H 7.0, O 37.0, N <0.2 (theoretical C 55.9, H 6.9, O 37.2). This corresponds to a purity of over 99.5%. The solid PHB had a fibrous consistency. The yield was 14.7 g (92%).

By way of comparison, when the above procedure was repeated using chloroform as the extraction solvent, while the yield was 16.6 g, the purity (before methanol washing) was only 88.6%.

EXAMPLE 7

An aqueous suspension of cells of Pseudomonas AML (NCIB 9133) made by fermentation of methanol in an aqueous medium and containing  $20 \text{ g l}^{-1}$  total biomass solids of which 28% was PHB was extracted by contact with 1,2-dichloroethane by the technique of Example 6 save that, after concentration to a cell cream containing  $120 \text{ g l}^{-1}$  biomass solids, the cells were disrupted by milling in a Dynomill at a throughput rate of  $15 \text{ l hr}^{-1}$  at an inlet temperature of  $20^{\circ}\text{C}$  and an outlet temperature of  $40^{\circ}\text{C}$ .

The PHB produced had a purity (before methanol washing) of 93.2% and the yield was 77%.

#### EXAMPLE 8

Example 7 was repeated using an aqueous suspension of cells as used in Example 1 concentrated to a cell cream of  $120 \text{ g l}^{-1}$  biomass solids by centrifugation. The yield of crude PHB (purity 93.2%) was 63.8%. The level of this yield was partly as a result of the fact that the separated lower layer had a volume of only 750 ml, ie only 75% of the 1,2 dichloroethane was separated from the emulsion. If all the 1,2-dichloroethane had been separated, calculation shows that the yield of PHB would have been about 85%.

#### EXAMPLE 9

Example 6 was repeated except that after mixing the cell cream (which had a pH of 7 and had been stored for a few weeks after fermentation) and 1,2-dichloroethane in the Silverson blender, the emulsion was allowed to settle under natural gravity for 30 minutes instead of cooling and separation by centrifugation. During this time the emulsion separated into an upper aqueous layer containing the cell debris and a lower layer of a solution of PHB in 1,2-dichloroethane. The two layers were separated by decanting. The recovered lower layer had a volume of only 170 ml. Even after standing for 1 hour the volume of the separated lower layer was only 420 ml. To obtain rapid separation with this system (at pH 7) it was necessary to cool the emulsion and centrifuge it for 15 minutes at  $15^{\circ}\text{C}$ .

#### EXAMPLE 10

Example 9 was repeated but the pH of the cell cream was adjusted to various values before contacting the suspension with 1,2-dichloroethane. The amount of 1,2-dichloroethane solution separating on standing for 30 and 60 minutes was as follows

pH	Volume of 1,2-dichloroethane separated (ml)	
	after 30 min	after 60 min
4	0 (stable emulsion)	30
4.5	410	-
5	650	770
5.5	640	-
6	290	700
7	170	420
8	60	300
9	50	270
10	30	-

The isoelectric point of the cell cream was also determined and found to be at pH 5.

#### EXAMPLE 11

Example 9 was repeated with a freshly fermented sample of an Azotobacter chroococcum (NCIB 9125) cell cream of isoelectric point at pH 5 in which the cells contained about 40% PHB. The pH was adjusted to 5 prior to contacting the cell cream with 1,2-dichloroethane. The aqueous and 1,2-dichloroethane layers separated rapidly under natural gravity and after only 10 minutes 850 ml of the 1,2-dichloroethane layer had separated and was recovered. The PHB was precipitated therefrom and washed, as in Example 6. The yield of PHB (purity 99.5%) was 84%.

#### EXAMPLE 12

An aqueous suspension of cells of Pseudomonas AML (NCIB 9133) was made by aerobic fermentation of methanol in an aqueous medium at pH 7.0. The suspension contained  $20 \text{ g l}^{-1}$  total biomass solids of which 30% was PHB. The suspension was concentrated by means of a centrifuge to a cell cream containing  $80 \text{ g l}^{-1}$  biomass solids. The cells were disrupted by milling the cell cream in a Dynomill at a throughput rate of  $12 \text{ l hr}^{-1}$  at an inlet temperature



of 20°C and an outlet temperature of 40°C. The isoelectric point of the resultant broken cells was determined and found to be pH 4.75.

The pH of the suspension of broken cells was adjusted  
5 to 4.75 and 500 ml of the suspension was added to 1 litre of 1,2-dichloroethane. The mixture was mixed for 10 minutes using a Silverson (RTM) blender, model L2R at a low mixing speed. The resultant emulsion was then allowed to settle.  
After 10 minutes 800 ml of 1,2-dichloroethane had separated. The  
10 PHB was then separated from the 1,2-dichloroethane by the procedure of Example 5. The yield of PHB was 78%.

When this experiment was repeated but without any pH adjustment, on mixing the suspension of broken cells and 1,2-dichloroethane, a relatively stable emulsion was formed that could  
15 be separated only after centrifugation.

#### EXAMPLE 13

To compare the effectiveness of various solvents as extraction solvents 500 ml of Azotobacter chroococcum (NCIB 9125) cell cream of biomass content 50 g l<sup>-1</sup> and pH 5.0 was poured at 20°C  
20 into 1 litre of the solvent under examination. The resulting mixture was mixed in a Silverson blender model L2R for 10 minutes and then allowed to separate under gravity. The amount of solvent separated after 30 and 60 minutes was measured. After separation, the solvent layer, herein termed "syrup", was syphoned off and its  
25 solids content determined by evaporation of a sample to dryness. The PHB content of the syrup was also determined by precipitation into a water/methanol mixture as in Example 6.

The procedure was repeated substituting a period of 15 minutes under conditions of reflux for the blending in the Silverson  
30 blender.

The results are shown in the following table

Solvent	cold extraction				reflux extraction	
	% solvent recovered		% solids content of syrup		% solids content of syrup	
	30 min	60 min	total	PHB	total	PHB
1,2-dichloroethane	55	75	1.15	0.89	1.32	0.92
chloroform*	0	0	1.27	0.92	1.35	0.95
dichloromethane*	12	35	1.51	0.95	1.07	0.69
1,1,1-tri-chloroethane	45	60	0.8	ND	0.85	ND
1,1,2-tri-chloroethylene	45	50	0.12	ND	0.22	ND
1,1,2,2-tetra-chloroethane	85	90	0.01	ND	0.05	ND
pyridine*	0	0	0.01	ND	-	-
1,2-propylene carbonate*	8	15	0.01	ND	ND	ND

ND = none detected

\* in view of the low separations on standing, the emulsion was centrifuged to obtain the samples for determination of the total solids and PHB contents.

To illustrate the solubility characteristics of PHB in various solvents, re-resolution of the precipitated PHB obtained by the cold extraction with chloroform, dichloromethane and 1,2-dichloroethane was attempted by mixing the precipitated PHB after drying with a fresh quantity of the original cold solvent. The precipitated PHB redissolved in cold chloroform or dichloromethane but was not soluble in cold 1,2-dichloroethane.

PA/CG/MP  
11 January 1980

1. A process for the extraction of poly-3-hydroxybutyric acid (PHB) from an aqueous suspension of PHB-containing bacterial cells comprising introducing said suspension in finely divided form into a current of gas heated to a temperature of at least 100°C to evaporate the water from the suspension, collecting the resultant dried bacterial cells, extracting the PHB therefrom by contact with an extraction solvent which is a liquid that is a solvent for the PHB in the bacterial cells and separating the extraction solvent having the PHB dissolved therein from the bacterial cell residue.
2. A process according to claim 1 wherein, prior to contact of the dried cells with the extraction solvent, the dried cells and contacted with a liquid in which the PHB is not soluble but in which lipids and/or pigment (if any) in the bacterial cells is soluble.
3. A process according to claim 2 in which the liquid in which PHB is not soluble is acetone or methanol.
4. A process according to any one of claims 1 to 3 in which the gas is heated to a temperature in the range 100 to 500°C.
5. A process according to any one of claims 1 to 4 in which the extraction solvent is a partially halogenated hydrocarbon.
6. A process according to claim 5 in which the extraction solvent is 1,2-dichloroethane, dichloromethane, or chloroform.
7. A process according to any one of claims 1 to 6 in which the dried cells are contacted with the extraction solvent at a temperature above 40°C.
8. A process according to any one of claims 1 to 7 in which the weight of extraction solvent is 10 to 100 times the cell dry weight.
9. A process for the extraction of PHB from bacterial cells containing PHB comprising contacting the bacterial cells with 1,2-dichloroethane and separating the PHB-containing solvent phase from the bacterial cell residue.
10. A process according to claim 9 in which an aqueous suspension of the cells is subjected to a cell disruption step prior to contact of the cells with 1,2-dichloroethane.
11. A process according to any one of claims 9 and 10 in

which the 1,2-dichloroethane is contacted with an aqueous suspension of the bacterial cells.

12. A process according to claim 10 or claim 11 in which the cells are contacted with 1,2-dichloroethane at a temperature between 10°C and 40°C.

13. A process according to claim 11 wherein the pH of the aqueous suspension is adjusted to within 0.5 units of pH of the isoelectric point of the cells, or of the disrupted cells if the cells are subjected to a cell disruption step, prior to contact with the 1,2-dichloroethane.

14. A process according to any one of claims 1 to 14 in which the PHB is separated from the extraction solvent by precipitation into a liquid in which PHB is insoluble and with which the extraction solvent is miscible.



DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D, X	<p>US - A - 3 044 942 (J.N. BAPTIST et al., W.R. GRACE &amp; CO.)</p> <p>* Column 2, lines 57-65; examples 7,8; claims 1,3,4,14,15,16,17</p> <p>--</p>	1-3,5,6,8,14	<p>C 12 P 7/62</p> <p>C 08 G 63/72</p>
D, X	<p>US - A - 3 036 959 (J.N. BAPTIST et al., W.R. GRACE &amp; CO.)</p> <p>* Column 2, lines 41-60; examples 9,10; claims 1,13,16,17</p> <p>--</p>	1-3,5,6,8,14	
A	<p>MACROMOLECULES, vol. 9, no. 5, September-October 1976, pages 774-80 S.AKITA et al.: "Solution Properties of Poly(D-<math>\beta</math>-hydroxybutyrate). I. Biosynthesis and Characterization"</p> <p>* Page 775, Viscometry; Osmometry, Optical Measurement</p> <p>----</p>		<p>TECHNICAL FIELDS SEARCHED (Int. Cl.)</p> <p>C 12 P 7/62</p> <p>C 08 G 63/06</p> <p>63/72</p> <p>C 12 N 1/32</p>
			<p>CATEGORY OF CITED DOCUMENTS</p> <p>X: particularly relevant</p> <p>A: technological background</p> <p>O: non-written disclosure</p> <p>P: intermediate document</p> <p>T: theory or principle underlying the invention</p> <p>E: conflicting application</p> <p>D: document cited in the application</p> <p>L: citation for other reasons</p>
<p>X The present search report has been drawn up for all claims</p>			<p>&amp; member of the same patent family.</p> <p>corresponding document</p>
Place of search The Hague		Date of completion of the search 05-06-1980	Examiner DESCAMPS